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Mary Ann Liebert, Inc.

Coreceptor Utilization of HIV Type 1 Subtype E Viral Isolates from Thai Men with HIV Type 1-Infected and Uninfected Wives

UTAIWAN UTAIPAT,[1,2] ANN DUERR,[3] DONNA L. RUDOLPH,[1] CHUNFU YANG,[1] SALVATORE T. BUTERA,[4] DAVIS LUPO,[4] TRACY PISELL,[4] ARUNRAT TANGMUNKONGVORAKUL,[2] NUONCHUEN KAMTORN,[5] NIWES NANTACHIT,[2] TIPPAVAN NAGACHINTA,[3] VINAI SURIYANON,[2] VALERIE ROBISON,[2] KENRAD E. NELSON,[6] NOPPORN SITTISOMBUT,[1] and RENU B. LAL[1]

ABSTRACT

HIV-1 coreceptors CCR5 and CXCR4 play an important role in viral entry and pathogenesis. To better understand the role of viral tropism in HIV-1 transmission, we examined the coreceptor utilization of viral isolates obtained from men enrolled in a study of heterosexual transmission in northern Thailand. Viral isolates were obtained from HIV-1-positive males who had either HIV-1-infected spouses (RM; $n = 5$) or HIV-1-uninfected spouses (HM; $n = 10$). Viral isolates from 1 of the 5 RM males and 2 of the 10 HM males were CCR5 tropic, whereas isolates from 3 RM males and 6 of the HM male isolates were CXCR4 tropic. Of the nine X4-tropic isolates, seven also used at least one of the following coreceptors: CCR8, CCR1, CCR2b, or CX3CR1, and none employed CCR5 as an additional coreceptor. More importantly, three isolates, RM-15, HM-13, and HM-16 (one from a transmitter and two from nontransmitter), did not infect GHOST4.cl.34 cells expressing any of the known coreceptors. Further analysis using MAGI-plaque assays, which allow visualization of infected cells, revealed that RM-15 had low numbers of infected cells in MAGI-R5 and MAGI-X4 cultures, whereas HM-13 and HM-16 had high levels of plaques in MAGI-X4 cultures. Replication kinetics using activated lymphocytes revealed that these three isolates replicated in $CCR5^{+/+}$ as well as $CCR5^{-/-}$ peripheral blood mononuclear cells, suggesting that these isolates did not have an absolute requirement of CCR5 for viral entry. All three isolates were sensitive to the X4-antagonistic compounds T-22 and AMD3100. Analysis of the C2V3 region did not reveal any significant structural differences between any of the Thai subtype E isolates. Thus, there was no association between the pattern of coreceptor usage and transmissibility among these subtype E HIV-1 isolates.

INTRODUCTION

HIV-1 ESTABLISHES A PERSISTENT INFECTION in cells expressing CD4, with T cells and macrophages being the principal target cells.[1–3] The specificity of the interaction between the viral envelope glycoprotein and the cellular receptor complex, which consists of CD4 and the seven-transmembrane-spanning chemokine receptor proteins, determines the cellular tropism of the virus.[2,3] It is now well established that the CC chemokine (β-chemokine) receptor CCR5 (R5) mediates the binding and entry of macrophage-tropic/non-syncytium-inducing (NSI) isolates, whereas the CXC chemokine (α-chemokine) receptor, CXCR4 (X4), mediates the entry of T cell-tropic/syncytium-inducing (SI) isolates.[4–9] The coreceptors, CCR5 and CXCR4 remain the predominant coreceptors used by HIV-1 regardless of distinct viral subtypes.[10,11]

[1]HIV Immunology and Diagnostics Branch, DASTLR, Centers for Disease Control and Prevention, Atlanta, Georgia.
[2]Research Institute for Health Sciences, and Department of Medicine and Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
[3]HIV Section, Women's Health and Fertility Branch, Centers for Disease Control and Prevention, Atlanta, Georgia.
[4]HIV Retroviruses Diseases Branch, DASTLR, Centers for Disease Control and Prevention, Atlanta, Georgia.
[5]Blood Bank Service, Chiang Mai University Hospital, Chiang Mai, Thailand.
[6]Department of Epidemiology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland.

A major role for CCR5 in HIV entry and transmission was established when persons with a Δ 32-bp deletion in the CCR5 gene were shown to be resistant to infection by certain HIV-1 isolates.[1,2,12,13] Further studies have established that viral entry can be blocked by coreceptor ligands or by CCR5- and CXCR4-antagonistic compounds.[14–19] In addition, differential coreceptor use has also been correlated with the stage of disease, that is, CCR5-tropic (R5) strains are predominantly present during the asymptomatic phase of infection, whereas dual-tropic (R5X4) strains are frequently isolated from patients with symptomatic infection or AIDS.[20–25] Thus, coreceptor utilization plays an important role both in HIV transmission and HIV-1 disease progression.

While sexual transmission remains the predominant route of HIV transmission worldwide, little is known about the ability of some individuals to resist HIV infection despite multiple and repeated exposure to HIV. Resistance to HIV infection is frequently observed in several categories of HIV-exposed persons including neonates of HIV-infected mothers, commercial sex workers, and partners/spouses of HIV-seropositive individuals.[26–31] Several studies have suggested that some of these HIV-exposed, uninfected persons have evidence of HIV-specific immunity, including HIV-specific humoral IgA antibodies, antigen-specific helper T and cytotoxic T cell responses, and antigen-driven cytokine and chemokine induction.[26,28,29,30,32] While these studies have established that host factors are important elements for resistance to infection, issues related to viral tropism have not been studied.

In the present study, we have estimated the cellular tropism of viral isolates derived from HIV-1-infected men enrolled in an HIV transmission study of heterosexual couples in Thailand. We sought to determine the difference in overall coreceptor usage of viral isolates from transmitting males (men with an HIV-positive spouse) or nontransmitting males (men with an HIV-negative spouse).

MATERIALS AND METHODS

Subjects

All subjects were drawn from an HIV-1 transmission study performed in northern Thailand. HIV-seropositive heterosexual male blood donors were identified at the blood banks of the Thai Red Cross or the Chiang Mai University Hospital, Chiang Mai, and Lampang Provincial Hospital, Lampang, from March 1989 through September 1995; HIV-1-infected men and their regular female partners were enrolled.[32] Only women whose sole risk for HIV infection was sexual exposure to her HIV-infected partner were eligible. Forty-seven percent of the women ($n = 590$) were HIV infected at the enrollment visit; for the vast majority of women, the length of exposure to an HIV-infected partner could not be defined precisely. Among the 53% who were HIV negative, however, we were able to identify a subset of 21 women who had a documented high-level exposure to an HIV-infected partner. Each of these 21 women had been married to a man who was documented to be HIV-1 infected for at least 2 years at enrollment, they reported at least twice weekly sexual intercourse during the previous 2 years, and they reported no condom use or condom use less than 25% of the time (termed HEPS; highly HIV-1 exposed, persistently seronegative). In addition, two women with commercial sex work history and who were also married to HIV-infected men were enrolled. We also identified a second group of women who seroconverted to HIV-1 after a short exposure to HIV-1 (RS; rapid seroconverter). These 17 women were HIV infected at enrollment after an exposure of 1 year or less to an HIV-infected husband. Wives in both groups reported no risk factor other than having unprotected sex with their HIV-infected husbands and there were no obvious differences in the frequency of sexual intercourse and history of sexually transmitted diseases between the two groups.

In 1997–1998, the 21 HEPS women returned for an additional blood draw and questionnaire; a blood sample was obtained from 13 of their male partners (8 were deceased or separated from their wives), who were considered nontransmitters. Virus isolations were attempted from 10 (no peripheral blood mononuclear cells [PBMCs] available for 3 men) of these 13 male subjects (HEPS men or HM), and the 8 subjects from whom we were able to isolate virus are part of the present study. In addition, we enrolled the HIV-1-infected husbands of the two seronegative women who gave a history of past commercial sex work. Although we were able to document sexual exposure of less than 12 months to these husbands, both men appeared to have been infected for a long time as both had AIDS-related conditions, and had low $CD4^+$ cell counts at enrollment (HM-11 and HM-13). As part of the original substudy, samples were also obtained from 9 of the men associated with the 17 RS women, who seroconverted rapidly (no PBMCs were available for 8 men); these men were considered transmitters (rapidly seroconverting men or RM). Virus isolations were attempted from eight of these men and five of the isolates are part of the present study.

All male participants in both groups were known to be infected for 9 to 98 months at the time of enrollment. For each husband, the absolute $CD4^+$ cell count, the rate of $CD4^+$ cell decline, and viral load (Roche Molecular Systems, Indianapolis, IN) at the time of blood draw for virus isolation is shown in Table 1. The mean absolute $CD4^+$ cell levels of the transmitter (RM) and nontransmitter (HM) groups were 114/μl and 189/μl, respectively. There was no difference in the mean viral load between transmitter and nontransmitter (data not shown).

Virus isolation and expansion

PBMCs were isolated by standard Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. HIV-1 isolations were carried out by coculturing PBMCs from infected husbands with phytohemagglutinin (PHA)-stimulated cells derived from uninfected Thai blood bank donors, essentially as described previously.[21,31] Viral replication was monitored twice a week for 3 weeks by measuring reverse transcriptase activity in viral pellets derived by centrifugation of culture fluid at 16,000 rpm in a refrigerated microcentrifuge for 1.5 hr. Samples that yielded two consecutive positive reverse transcriptase results were expanded for an addition 1–2 weeks; culture supernatants with the highest reverse transcriptase activity were pooled, filtered through a 0.22-μm pore size membrane, aliquoted, and stored at −70°C until use. Viral isolates

TABLE 1. CORECEPTOR USAGE OF HIV-1 SUBTYPE E ISOLATES FROM THAI MEN WITH HETEROSEXUAL RISK

Isolate	Known duration of HIV-1 seropositivity (months)	Absolute $CD4^+$ cell count (cells/μl)	Rate of $CD4^+$ cell decline (cells/month)	Viral load (copies/ml)	Infection of chemokine receptor-expressing GHOST4 cells[a]									
					R1	*R2b*	*R3*	*R4*	*R5*	*R8*	*X4*	*Gpr15*	*STRL3*	*X3CR1*
Transmitters														
RM-09	25	192	6.7	2.6×10^4	−	−	−	−	+	−	−	−	−	−
RM-15[b]	89	112	9.3	1.1×10^3	−	−	−	−	−	−	−	−	−	−
RM-16	84	113		5.8×10^4	−	−	−	−	−	−	+++	−	−	−
RM-02	55	79	3.7	1.9×10^5	+	−	−	−	−	+	+++	−	−	−
RM-07	68	74	7.8	3.1×10^4	+	−	−	−	−	+	+++	−	−	−
Nontransmitters														
HM-03	92	369	6.4	3.7×10^4	−	−	−	−	+	−	−	−	−	−
HM-12	80	253	3.2	8.0×10^4	−	−	−	−	+++	−	−	−	−	−
HM-05	71	65	8.6	1.0×10^5	+	−	−	−	−	−	+	−	−	−
HM-01	82	567	6.6	1.4×10^5	−	−	+	−	−	−	+	−	−	−
HM-04	86	185	9.12	1.1×10^5	−	−	−	−	−	−	+++	−	−	−
HM-16[b]	94	161	11.9	1.7×10^4	−	−	−	−	−	−	−	−	−	−
HM-14	82	36	NA	2.6×10^5	+	+	−	−	−	+++	+++	−	−	−
HM-19	98	217	6.1	4.4×10^4	+	−	−	−	−	+	+++	−	−	−
HM-13[b]	12	7	4.2	2.7×10^4	−	−	−	−	−	−	−	−	−	−
HM-11	9	28	NA	8.5×10^4	−	−	−	−	−	+++	+++	−	−	−

Abbreviation: NA, data not available.

[a]Level of p24 antigen (pg/ml) at day 11 postinfection: −, <50 pg/ml; +, 50–125 pg/ml; ++, 125–250 pg/ml; +++, >250 pg/ml.

[b]Infection of U87-R5 and U87-X4 was also negative.

were successfully obtained from 10 of the 12 HM men and from 5 of the 8 RM men (Table 1).

HIV-1 infection of PBMCs

The CCR5 genotype of PBMCs was determined by polymerase chain reaction (PCR),[31] using primers 5′-CTTCATTACACCTGCAGCTCTC-3′ and 5′-CTCACAGCCCAGTGCGACTTCTTCT-3′, which flank the 32-bp deletion. Samples from donors with CCR5 wild type ($CCR5^{+/+}$) generate PCR fragments of 184 bp, whereas those with the homozygous deletion ($CCR5^{-/-}$) result in a 152-bp product. PBMCs from individuals either wild type or homozygous for the 32-bp deletion in CCR5 were depleted of $CD8^+$ T cells by incubation with magnetic beads coated with anti-CD8 antibody (Dynabeads; Dynal, Lake Success, NY), according to the manufacturer instructions. In some experiments, PBMCs from HIV-negative women from Thailand (spouse's cells) or blood donors were used. The CD4-enriched PBMCs were plated in 24-well plates at 2×10^6 cells/well in a total volume of 2 ml of RPMI supplemented with 10% fetal calf serum (FCS) and 10% interleukin 2 (IL-2) (C-RPMI) and stimulated with PHA (0.1%) for 2–3 days. Unless specified otherwise, the PHA-stimulated $CD4^+$ cells were infected with various HIV-1 isolates at 40,000 reverse transcriptase (RT) counts/ml as described previously.[21] Cell-culture adapted, T cell-tropic (LAI) and macrophage-tropic (BAL) strains of HIV-1 subtype B were used as controls. After infection, half of the culture supernatant was collected every other day for 14 days, and replaced with C-RPMI. Levels of RT were determined by standard assay or enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter/Immunotech, Westbrook, ME)-determined p24 antigen in the culture supernatant. In some experiments, infections were carried out in the absence or presence of X4 antagonistic compounds T-22 (0.3 μM) and AMD3100 (200 ng/ml) as described previously.[16,17,33]

HIV receptor-expressing cell lines and infection

GHOST4.cl.34, U87, and U373-MAGI cell lines expressing various coreceptors were obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD). GHOST4.cl.34 cell lines expressing CD4 together with either CCR1, CCR2B, CCR3, CCR4, CCR5, CCR8, CXCR4, CX3CR1, gpr15, or STRL33 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, puromycin (1 μg/ml), hygromycin B (100 μg/ml), and G418 (500 μg/ml) and infected with HIV-1 as described previously.[11] Briefly, GHOST4.cl.34 cells were plated at 4×10^4 cells per well in the 24-well culture plate and infected with 40,000 RT counts of virus stocks. In some experiments, infections were carried out with 100,000 counts. After 16–18 hr of incubation with an individual isolate, cells were washed three times with phosphate-buffered saline, and the cultures were further maintained in 2 ml of supplemented DMEM. Culture supernatant was collected every 3–4 days and tested for HIV p24 antigen by ELISA. Wells with p24 levels of 50 pg/ml or higher were considered positive for HIV replication. Cell culture-adapted, T cell-tropic (LAI) and macrophage-tropic

(BAL) strains of HIV-1 subtype B were used as controls. U87.CCR5 and U87.CXCR4 cell lines were infected in a similar fashion. All infections were repeated at least three times, and representative experiments are shown in the figures. The U373-MAGI-CCR5 and U373-MAGI-CXCX4 cell lines, expressing CCR5 and CXCR4, respectively, are U373-MAGI cell derivatives that also express the human CD4 gene and the bacterial β-galactosidase (β-Gal) gene under the control of the HIV-1 long terminal repeat (LTR).[34] HIV infection of MAGI cells was carried out in multiple dilutions, and infection resulted in blue staining of the infected cell nuclei after the addition of β-Gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]).[34] The cell surface staining for coreceptor expression was carried out by flow cytometry analysis, using antibodies to CD4 (Beckton Dickinson, San Jose, CA) and to CCR5 and CXCR4 (Pharmingen, San Diego, CA).

Sequencing and phylogenetic analysis

HIV-1-infected cells from day 4 or day 7 of culture were lysed by treatment with lysis buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.45% Nonidet P-40 [NP-40], 0.45% Tween 20, and proteinase K [0.1 mg/ml], pH 8.3) at 95°C at 56°C for 2 hr. After the proteinase K was heat inactivated, the C2V3 region of the envelope gene was amplified by 35 cycles of PCR with 10 μl of the lysate, using primers

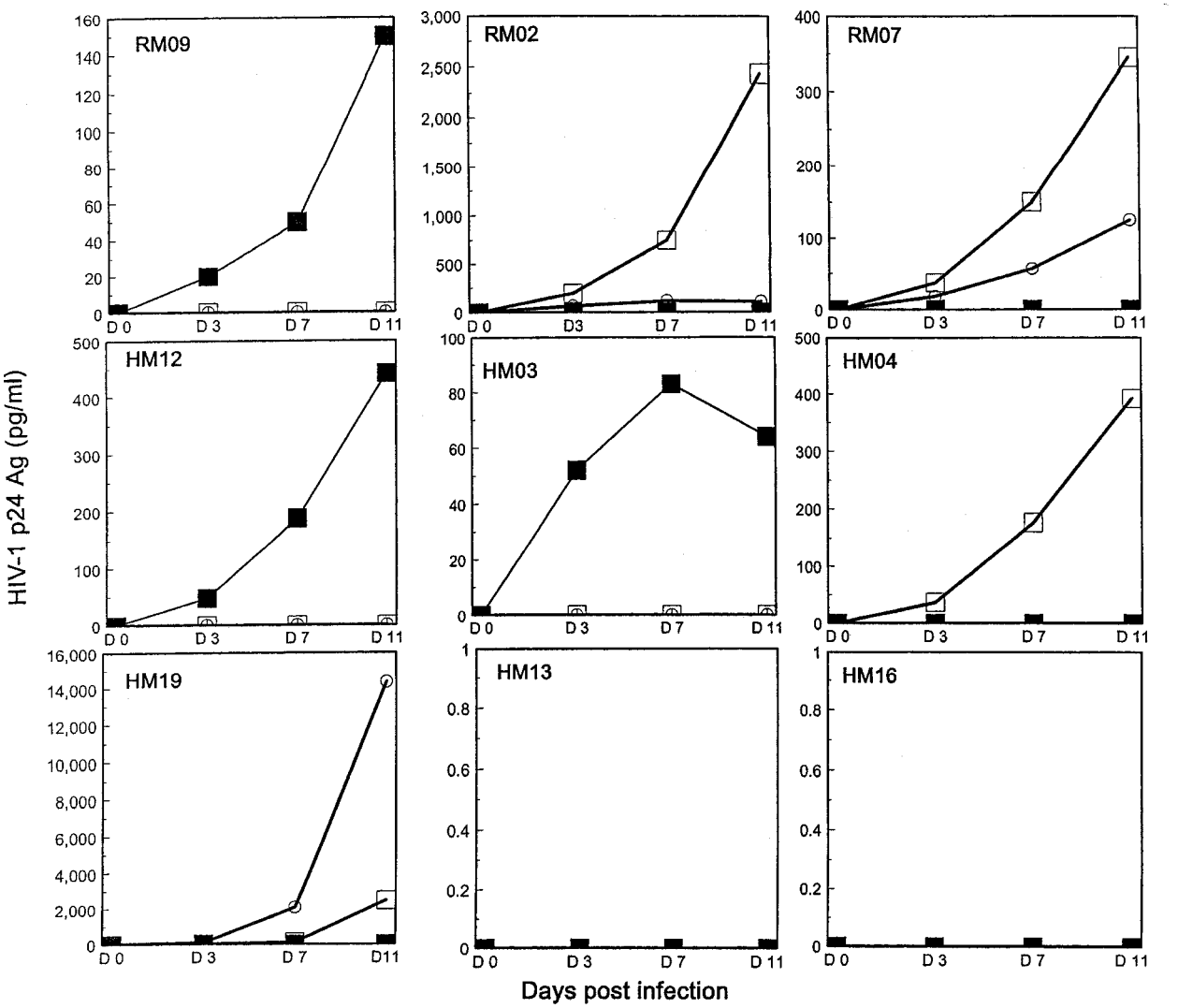

FIG. 1. Replication kinetics of p24 production by HIV-1 isolates from Thailand in GHOST4 cells coexpressing CCR5 (■), CXCR4 (□), and CCR8 (○). *Top*: HIV-1 isolates derived from Thai men who transmitted HIV-1 to their wives (transmitters: RM-09, RM-02, and RM-07). *Middle* and *bottom*: HIV-1 isolates derived from Thai men who did not transmit the virus to their wives (nontransmitters: HM-12, HM-03, HM-04, HM-19, HM-13, and HM-16). The values given represent p24 antigen (picograms per milliliter) present in the culture supernatant on days 0, 3, 7, and 11 from various GHOST cell lines infected with the same dose of input virus. Two representative isolates (HM-13 and HM-16) that did not infect any of the GHOST cell lines coexpressing CD4 and the known coreceptors are shown.

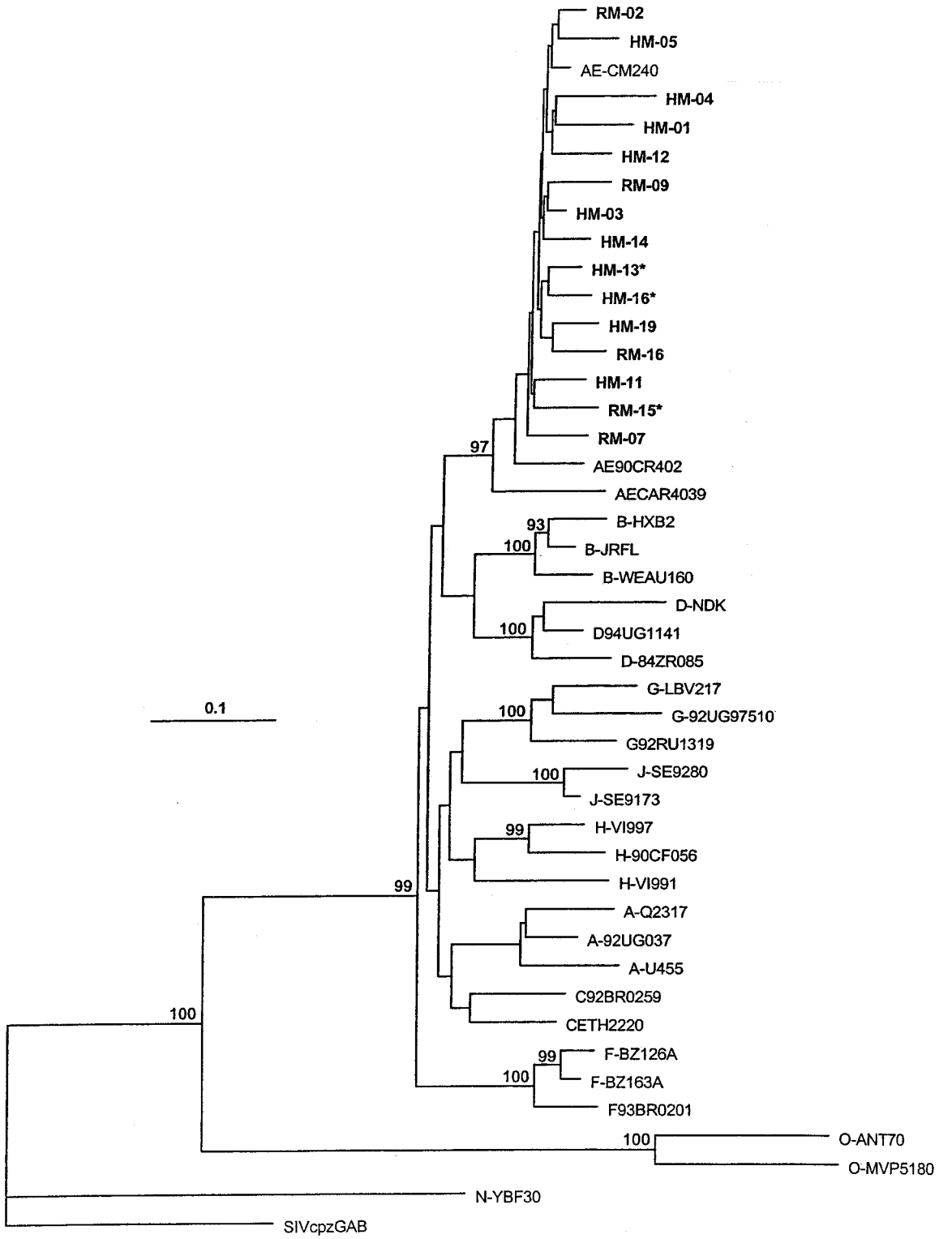

FIG. 2. Phylogenetic relationship of Thai isolates with representative HIV-1 group M subtypes A–G. The tree was constructed from partial *env* nucleotide sequences in the C2V3 region (consensus alignment of 364 bp). The phylogenetic relationships were determined by the neighbor-joining method as described in Materials and Methods. The numbers on the nodes represent the percentage bootstrap samples with which the cluster to the right is supported; only values over 80% are shown. The tree was rooted by using SIVcpzGAB as an outgroup. The isolates analyzed in this study are in boldface, and the three isolates (HM-13, HM-16, and RM-15) with unknown coreceptor specificities are indicated by asterisks.

TABLE 2. INFECTION OF MAGI-X4 AND MAGI-R5 CELL LINES WITH SELECTED THAI ISOLATES

Isolate	*Coreceptor utilization*	*Plaques/150 μl*		*Ratio X4/R5*
		MAGI-X4	*MAGI-R5*	
LAI	X4	293[a]	0	≫200
BAL	R5	90[a]	248	0.37
RM-07	X4, R8	725	2	362
RM-15	?	11	5	2.2
HM-14	X4, R1, R2b, R8	2310	10	231
HM-13	?	212	7	30
HM-16	?	285	7	41

[a]Viral stocks for LAI and BAL were used at 0.5 and 0.3 μl, respectively.

5′JH-44 (5′-ACA GTR CAR TGY ACA CAT GG-3′) and JH35M (5′-CAC TTC TCC AAT TGT CCI TCA-3′). The nested PCR was performed with 5 μl of the primary PCR products, using primers JH33 (5′-CTG TTI AAT GGC AGI CTA GC-3′) and JH48 (5′-RAT GGG AGG RGY ATA CAT-3′). The nested PCR fragment was directly sequenced by cycle-sequencing and dye terminator methods with an automated DNA sequencer. DNA sequences were aligned and phylogenetic analysis was carried out by the neighbor-joining method.[35]

RESULTS

Coreceptor utilization of Thai isolates

We determined the range of chemokine coreceptors used by 15 HIV-1 primary isolates from northern Thailand by infection of GHOST4.cl.34 cell lines expressing human CD4 along with 1 of 10 individual chemokine receptors. Three isolates exclusively used CCR5, but most had broad coreceptor specificities

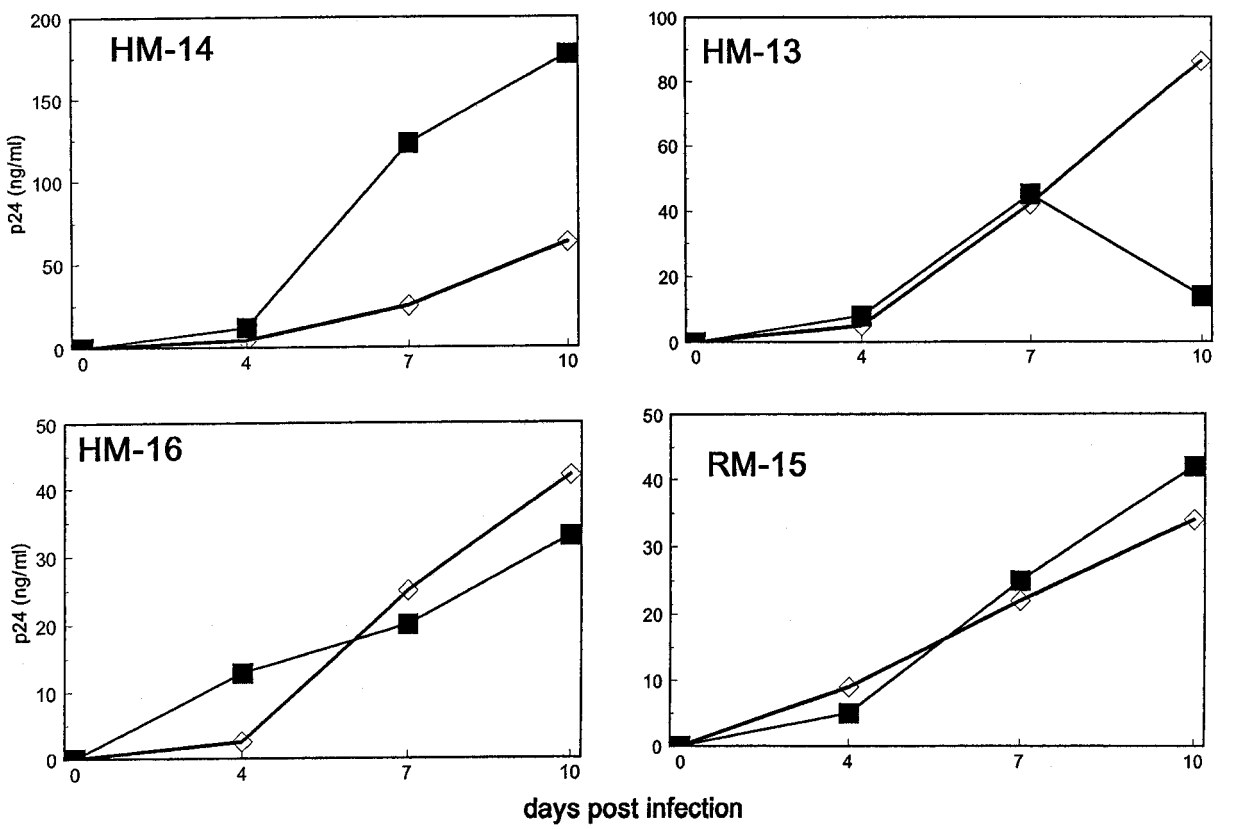

FIG. 3. Replication kinetics of viral isolates (HM-14, HM-13, RM-15, and HM-16) in CD8-depleted PBMCs derived from a donor wild type for CCR5 ($CCR5^{+/+}$; ■) or homozygous for a 32-bp deletion ($CCR5^{-/-}$; ◇). The values given represent p24 antigen (nanograms per milliliter) present in the culture supernatant on days 0, 4, 7, and 10. The HM-13, HM-16, and RM-15 isolates were capable of replicating in PBMCs, regardless of the CCR5 genotype of the donor PBMCs.

with utilization of CXCR4 and other coreceptors including CCR1, CCR2b, CCR3, CCR8, and CX3CR1 (Table 1). The kinetics of infection and replication for representative specimens are shown in Fig. 1. In contrast to previous studies in which most R5 isolates became dual tropic by acquiring X4 usage as HIV disease progressed,[20,21] most of the X4 viruses in the present study did not use CCR5. The presence of X4 viruses (60%) correlated well with duration of infection, since 12 of 15 subjects were known to have been infected for more than 4.5 years (Table 1). Three isolates (RM-15, HM-13, and HM-16; Table 1) repeatedly failed to infect only GHOST4.cl.34 cell lines or the U87.CCR5 or U87.CXCR4 cell line (data not shown).

We next examined the association between chemokine coreceptor usage and the transmission or nontransmission of HIV-1 from the infected men to their spouses. One of the limitations of the study is that viral isolates were not derived close to the transmission time and there is no knowledge of the viral isolates from the infected spouse. The frequency of CCR5 usage for isolates derived from men who had transmitted virus to their spouse (1 of 5, 20%) and isolates derived from men who did not transmit HIV-1 to their spouses (2 of 10, 20%) were the same, as was the frequency of CXCR4 usage (3 of 5 vs. 6 of 10, 60%) (Table 1). The men infected with the three viruses with potential unknown coreceptor usage (RM-15, HM-13, and HM-16) had no difference in absolute $CD4^+$ cell count or rate of $CD4^+$ cell decline (Table 1). Thus there appears to be no difference in the frequency of CCR5/CXCR4 usage among HIV-1 subtype E isolated from transmitting and nontransmitting husbands in this cohort. However, three isolates seem to have unique coreceptor specificities, since they were unable to use any of the known 10 coreceptors examined.

We next explored the possibility that the three viruses with unique or unknown coreceptor specificities might represent variants of HIV-1. The C2V3 region of the *env* gene was sequenced and phylogenetic analysis was carried out (Fig. 2). All isolates, including the three isolates with unique coreceptor specificities (RM-15, HM-13, and HM-16), were HIV-1 subtype E, as were the remainder of the 13 HIV-1 isolates (Fig. 2).

Replication efficiency of viral isolates

To further elucidate the coreceptor requirement for CCR5 and CXCR4 of the isolates that were untypeable by GHOST infection, the three strains were tested on U373-MAGI-R5 and U373-MAGI-X4 cell lines. Direct visualization of plaques revealed that all three exhibited lower numbers of infected cells

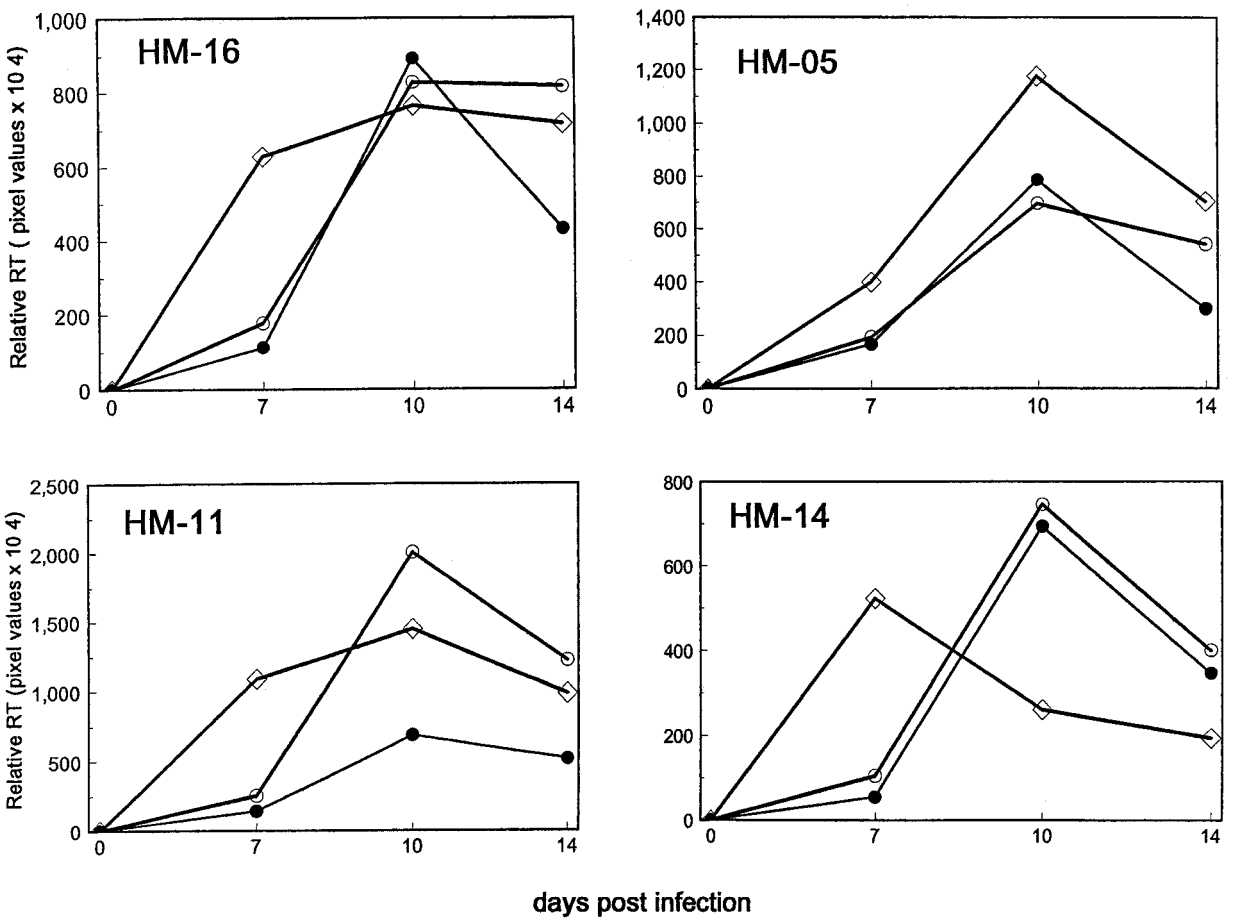

FIG. 4. Replication kinetics of viral isolates (HM-16, HM-05, HM-11, and HM-14) in CD8-depleted PBMCs derived from an HIV-negative spouse (●) or two Thai donors (○ and ◇). The values given are RT counts (pixel values $\times 10^4$) present in the culture supernatant on days 0, 7, 10, and 14. The HM-16 isolate is able to infect spouse cells, just as efficiently as other isolates.

in MAGI-X4 assays when compared with other X4 isolates, that is, RM-07 and HM-14 (Table 2). In addition, isolate RM-15 had low numbers of plaques in MAGI-R5 assays as well. Thus for Thai X4 viruses, the plaque-forming cells in the MAGI assay were at least 3- to 10-fold higher than those isolates with unknown coreceptor specificities (Table 2). To investigate whether the relative expression of the coreceptor on the cell surface of these cell types might be an important determinant for infection, we carried out fluorescence-activated cell sorting (FACS) analysis using antibodies to CCR5 and CXCR4. The FACS analysis of GHOST or U373-MAGI cells with monoclonal antibodies (MAbs) to CCR5 or CXCR4 revealed comparable expression of these receptors (mean fluorescence channel for CCR5 was 153 and 131, respectively, for GHOST-R5 and MAGI-R5; and 365 and 430, respectively, for GHOST-X4 and MAGI-X4) (data not shown). These data suggest that there might be some postentry block in viral infection when GHOST or U87 cells are used.

We next examined the replication kinetics of the three viral isolates (RM-15, HM-13, and HM-16), using PHA-activated $CD4^+$ cells from persons with functional CCR5 expression ($CCR5^{+/+}$) or with defective expression due to the presence of a homozygous 32-bp deletion ($CCR5^{-/-}$). Both the positive control (HM-14) and the three unique isolates (HM-13, HM-16, and RM-15) were capable of replicating in both $CCR5^{+/+}$ and $CCR5^{-/-}$ PBMCs to the same extent as two subtype E control strains (Fig. 3). Infection of PBMCs from the spouse was attempted for several isolates (HM-05, HM-01, HM-11, HM-14, HM-19, and HM-16). The unique isolate (HM-16) was capable of infecting his wife's cells just as efficiently as any of the other isolates with R5 or R5X4 tropism (HM-1, HM-5, HM-11, HM-14, and HM-19). Data on representative isolates (HM-16, HM-05, HM-11, and HM-14) are shown in Fig. 4. Taken together, these data suggest that these unique isolates did not have an absolute requirement for CCR5 for viral entry and that a coreceptor expressed on activated PBMCs can support viral entry of these three isolates.

To examine the possibility that infection of activated PBMCs might be mediated by X4, we used X4-specific antagonistic compounds AMD3100 and T-22.[15–17,19] The infections were

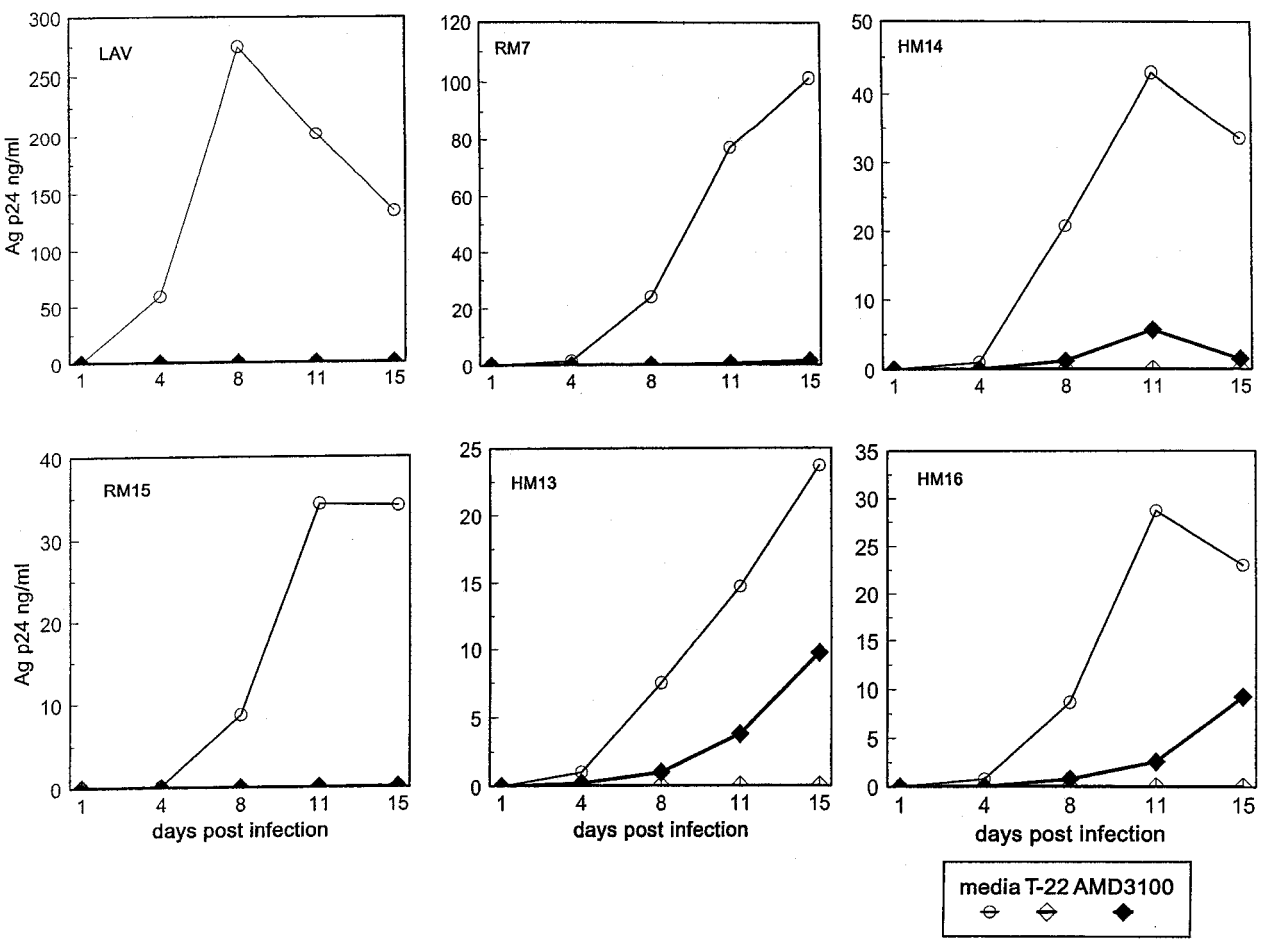

FIG. 5. Sensitivity of viral isolates to CXCR4-antagonistic compounds AMD3100 and T-22. Infection of CD8-depleted lymphocytes from a $CCR5^{-/-}$ donor was carried out with HIV-1 isolates (laboratory-adapted strain LAV; primary isolates RM-07, HM-14, RM-15, HM-13, and HM-16) in the absence (○) or presence (◇) of T-22 or in the presence of AMD3100 (◆). The values given represent p24 antigen (nanograms per milliliter) present in the culture supernatant on days 1, 4, 8, 11, and 15. All HIV-1 isolates tested were sensitive to T-22 (0.3 ng/ml) and AMD3100 (10 ng/ml).

TABLE 3. V3 LOOP AMINO ACID SEQUENCE ANALYSIS FROM TRANSMITTERS AND NONTRANSMITTERS

Isolate	*Coreceptor usage from GHOST cell infection*	*V3 sequence*[a]	*Net charge*
		1 11 20 30	
Consensus E		CTRPSNNTRT SI TIGPGQV FYRTGDIIGD IRKAYC	0
Transmitters			
RM-09	R5	.........R ..-N......L...P...... ..A...	−1
RM-15	R5	....FTKM.V .T-R.....-..K..S.T.. ....F.	+5
RM-16	X4	....Y.-.EI RM-.R...K.-.....N.V..	+4
RM-02	X4, R1, R8	.....T...K RM-.M...H.-..S..E.T..	+3.5
RM-07	X4, R8	.....T.I.. .A-R....R.-.H...A.S..	+5.5
Nontransmitters			
HM-03	R5	.......K.. RM-.....H.-.........N	+3.5
HM-12	R5	....-SM...R.-.......T.N ..R...	+2
HM-05	X4, R1	....F.Y.K. RM-.M.L.H.-....KE.V.. P...F.	+3.5
HM-01	X4, R3	....FA.... RMIH....RA-.F.A.GMT..	+5.5
HM-04	X4	....AGIR.. KV-.M...R.-..K..EV... ....H.	+3.5
HM-11	X4, R8	.I..FKKV.. .T-R....T.-.....S.S..	+7
HM-14	X4, R1, R2b, R8	.....TK.K. RM-.T...H.-..K.E..S.N	+5.5
HM-19	X4, R1, R8, X3CR1	....Y.-KK. R.-.M...R.-Y.Q..E.T.. ....H.	+2.5
HM-13	?	.....K-.KI G.-.R...R.-Y....E.T..	+5.5
HM-16	?	.....T.V.K RM-SM...H.-..S..Q.T..	+3.5

[a]Dot, amino acid identical to that in consensus E sequence; dash; deletion or insertion.

carried out in cells from a $CCR5^{-/-}$ homozygous donor in the absence or presence of AMD3100 and T-22. As expected, LAI, and two other control X4 virus, RM-07 and HM-14, were completely blocked by T-22 and AMD3100 (Fig. 5). Likewise, RM-15 was completely blocked by both inhibitors (Fig. 5), whereas HM-13 and HM-16 were completely blocked by T-22 and partially blocked by AMD3100 (Fig. 5). These results suggest that viral entry in activated PBMCs was most likely mediated via CXCR4 on activated cells or by an as yet unidentified coreceptor that is equally sensitive to T-22 or AMD3100.

We next searched for signature sequences that may be unique for the isolates in the V3 loop of the gp120 Env protein (Table 3). Overall, we found a significantly higher proportion of positively charged residues, which would be consistent with their preferential use of CXCR4.[11] The basic amino acid signature sequences (R or K) at positions 11 and 25 were observed in most X4 isolates. However, we did not find any specific difference between the overall charges of isolates derived from men who transmitted versus those who did not. These data are consistent with the observation that there was no difference in overall coreceptor utilization between the two groups. In addition, the three isolates (HM-13, HM-16, and RM-15) did not have any specific or obvious V3 sequence that might signify unique coreceptor utilization (Table 3).

DISCUSSION

The coreceptor utilization of viral isolates derived from men in a study of HIV sexual transmission among couples in Thailand revealed that while most isolates were R5 or X4 tropic, three of the isolates had limited capacity to use any of the known HIV-1 coreceptors expressed on GHOST cells. However, these isolates replicated well in activated PBMCs and were blocked by X4-antagonistic compounds, suggesting that they were able to use CXCR4 or an as yet unidentified coreceptor that is equally sensitive to T-22 and AMD3100 on activated T cells. Overall, there was no association between the pattern of coreceptor usage and transmission among this group of Thailand subtype E isolates.

In this study, three R5 isolates did not use other chemokine receptors, but most of the X4 isolates used one or several of the non-CCR5 receptors. Such broad coreceptor adaptations have previously been identified for other HIV-1 isolates.[9,11,19,36] In addition, differential tropism of the subtype E virus for dentritic cells was suggested[38]; however, subsequent studies could not confirm these findings.[27] Previous studies of the chemokine receptor usage of HIV subtype E viruses from Thailand revealed that most primary isolates were capable of using CCR5 as a coreceptor,[37,39] whereas in this study we found many that used CXCR4. The discrepancy may be due to the use of isolates derived from patients early in the epidemic (mostly 1992–1993) in previous studies, whereas we isolated HIV from our volunteers during 1997–1998, when most patients had $CD4^+$ cell levels below 200. Emergence of X4 has been shown to be associated with $CD4^+$ cell decline and faster disease progression.[23,25,40] In accordance with the results of studies of subtype B-infected subjects,[21] patients in this study with subtype E X4 viruses tended to have a lower mean $CD4^+$ cell level (151 vs. 274 cells/μl) and a faster $CD4^+$ cell decline (6.9 vs. 5.4 cells/μl per month) than did those infected with R5 viruses. Another unique observation in the present study is the

fact that most X4 viruses could not utilize R5. This finding is in contrast to previous reports indicating that most isolates that have acquired X4 usage retain dual coreceptor specificities.[20,21,24]

Another important finding of our study is the identification of three isolates that were incapable of infecting GHOST-CD4 cells that express each of the 10 chemokine receptors individually. However, the three isolates produced plaques in MAGI-X4 cells, albeit in low numbers, when compared with the number of plaques found in control X4 subtype E viruses. These data suggest there is some postentry block in viral replication when GHOST cells are used. Further, infection of activated lymphocytes from $CCR5^{+/+}$ and $CCR5^{-/-}$ individuals suggested that these unique isolates do not have an exclusive requirement for CCR5 for cell entry. However, the X4-antagonistic compounds T-22 and AMD3100[15–17,41] significantly blocked the entry of these unique isolates in PBMCs, suggesting that infection of PBMCs with these isolates was likely mediated via CXCR4 or via another coreceptor that is sensitive to the X4-antagonistic compounds. Thus, despite the lack of infection of GHOST cells coexpressing CXCR4 and the low-level infection of MAGI-X4 cells, the infection of PBMCs was most likely mediated via the CXCR4 coreceptor, or via an as yet unidentified coreceptor that is equally sensitive to T-22 and AMD3100. Because the V3 loop amino acid sequences of these unusual isolates are indistinguishable from those of other X4 subtype E viruses, it is possible that these viruses had diversified to the point that they require a higher level of CXCR4 on the surface of target GHOST-CD4 and U373-MAGI cells for effective infection. Whether these isolates require a certain threshold of X4 expression for productive infection of GHOST cells or a certain conformation of CD4–X4–gp120 binding complex for replication remains to be determined.

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Address reprint requests to:
Renu B. Lal
Immunopathogenesis Laboratory
HIV Immunology and Diagnostics Branch
CDC
1600 Clifton Road
Mail Stop D-12
Atlanta, Georgia 30333

E-mail: RBL3@cdc.gov